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The TOM Complex of Amoebozoans: the Cases of the Amoeba *Acanthamoeba castellanii* and the Slime Mold *Dictyostelium discoideum*

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Protein import into mitochondria requires a wide variety of proteins, forming complexes in both mitochondrial membranes. The TOM complex (translocase of the outer membrane) is responsible for decoding of targeting signals, translocation of imported proteins across or into the outer membrane, and their subsequent sorting. Thus the TOM complex is regarded as the main gate into mitochondria for imported proteins. Available data indicate that mitochondria of representative organisms from across the major phylogenetic lineages of eukaryotes differ in subunit organization of the TOM complex. The subunit organization of the TOM complex in the Amoebozoa is still elusive, so we decided to investigate its organization in the soil amoeba *Acanthamoeba castellanii* and the slime mold *Dictyostelium discoideum*. They represent two major subclades of the Amoebozoa: the Lobosa and Conosa, respectively. Our results confirm the presence of Tom70, Tom40 and Tom7 in the *A. castellanii* and *D. discoideum* TOM complex, while the presence of Tom22 and Tom20 is less supported. Interestingly, the Tom proteins display the highest similarity to Opisthokonta cognate proteins, with the exception of Tom40. Thus representatives of two major subclades of the Amoebozoa appear to be similar in organization of the TOM complex, despite differences in their lifestyle.

Key words: TOM complex; mitochondria; *Acanthamoeba castellanii*; *Dictyostelium discoideum*; phylogenesis; translocase of the outer membrane; Amoebozoa.

Introduction

Mitochondria are essential for cell function and survival. The proper function of mitochondria depends on protein import, regarded as extremely challenging due to mitochondrial architecture; i.e. the

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presence of the outer and inner membranes that form the borders for two aqueous compartments: the intermembrane space and matrix. As summarized by Schmidt et al. (2010), the import concerns all proteins of the outer membrane and the intermembrane space, as well as the majority of the inner membrane and matrix proteins. This results from the course of mitochondria formation during the evolution of an eukaryotic cell that consisted in gene transfer of an ancestor prokaryotic endosymbiont to the nucleus as well as in gene loss and emergence of new genes in the nucleus to control mitochondrial function (e.g. Cavalier-Smith 2010; Cavalier-Smith et al. 2014; Liu et al. 2011).

The protein import is mediated by a set of protein complexes located in both mitochondrial membranes (termed translocases) as well as in the intermembrane space and matrix but is initiated by the translocase of the outer mitochondrial membrane, known also as the TOM complex. The complex is regarded as a general entry gate for virtually all proteins imported into mitochondria (e.g. Becker et al. 2008; Dolezal et al. 2006; Hewitt et al. 2011; Lithgow and Schneider 2010; Mokranjac and Neupert 2009; Neupert and Herrmann 2007; Schmidt et al. 2010; Sokol et al. 2014; Varabyova et al. 2013; Walther et al. 2009). The translocase is a protein complex responsible for the imported protein recognition, translocation across or into the outer membrane, and for decoding of their targeting signals and subsequent sorting. Thus the TOM complex appears to play a fundamental role in the import process.

The subunit organization of the TOM complex, defined first for *Neurospora crassa* and *Saccharomyces cerevisiae* mitochondria (Ahting et al. 1999; Meisinger et al. 2001), is at present regarded as the canonical one. Moreover, available data allow the conclusion that the organization follows the scheme described for all translocases. It means that besides the subunit displaying channel-forming activity and being responsible for protein translocation, the complex contains subunits that regulate its structure and function. As summarized by Varabyova et al. (2013), in the case of the canonical TOM complex, the central subunit is Tom40, the channel-forming protein, whereas other proteins can be divided into two groups: (i) the core TOM subunits, which include the central receptor (Tom22) and the small Tom proteins (Tom5, Tom6, Tom7) regulating the complex dynamics; and (ii) the peripheral receptor subunits (Tom70, Tom20), which are more loosely associated with the complex and recognize different features and targeting signals within the imported proteins, which in turn initiate entry into a defined import pathway.

Interestingly, it has been proposed that mitochondrial protein import complexes, including the TOM complex, contain subunits formed by proteins common to all eukaryotes and additional subunits that have been added over time and regarded as common only to a particular eukaryotic lineage (Dolezal et al. 2006). Available data concerning representatives of different phylogenetic lineages indicate that only Tom40 is found in virtually all eukaryotes (Zarsky et al. 2012), whereas other subunits are definitely less conserved. In animals, the TOM complex subunit organization is very similar to the canonical one (e.g. Dolezal et al. 2006; Hewitt et al. 2011; Hoogenraad et al. 2002; Schneider et al. 2008), whereas in plants the complex does not contain orthologs of the canonical Tom20 and Tom70. Moreover, the plant ortholog of Tom22 is termed Tom9, while for Tom5 and Tom6 it is still discussed whether they are orthologous or analogous to the *S. cerevisiae* proteins (e.g. Murcha et al. 2014; Perry et al. 2008). In different protists, the TOM complex subunit organization usually differs distinctly from the canonical one but available data are not numerous and consistent.

Protists are a polyphyletic group of eukaryotic microorganisms defined in the past by exclusion of animals, fungi, and plants. In the recent classification proposed by the International Society of Protistologists (Adl et al. 2005), Eukaryota were divided into six supergroups: Chromalveolata, Excavata, Rhizaria, Amoebozoa (these four including protists only), Archaeplastida (including plants and some algae), and Opisthokonta (including animals, fungi and some protists). That classification was later discussed and refined by many authors (e.g. Adl et al. 2012; Cavalier-Smith et al. 2014; Keeling et al. 2005; Schilde and Schaap 2013). The Opisthokonta are most closely related to the Amoebozoa, which comprise a wide variety of amoeboid and flagellate organisms with single cells of various sizes that have adopted many different lifestyles and live in different environments. The Amoebozoa can be further be subdivided into the phyla Conosa, Lobosa, and probably Breviatea (e.g. Fiz-Palacios et al. 2013; Schilde and Schaap 2013).

Here we described the TOM complex of the amoeba *Acanthamoeba castellanii* and the slime mold *Dictyostelium discoideum*, representatives of the Lobosa and Conosa, respectively. Both species share properties with plant and animal cells and are known as particularly valuable research models for developmental biology and medicine (Annesley et al. 2014; Walker and Williams 2013). Previous

studies, based mainly on comparative sequence analysis, have suggested some Tom proteins; i.e. Tom40, Tom22 and Tom7 for *D. discoideum* (Barth et al. 2007; Dolezal et al. 2010; Maćasev et al. 2004) as well as Tom70, Tom40 and Tom7 for *A. castellanii* (Makiuchi et al. 2013; Wojtkowska et al. 2005, 2012). Taking into account the availability of the *A. castellanii* and *D. discoideum* genome and transcriptome data, we decided to combine in silico sequence analyses with experimental approaches to verify subunit organization of TOM complexes of these organisms.

Our results indicate that the *A. castellanii* and *D. discoideum* TOM complex contains five subunits, namely Tom70, Tom40, Tom22, Tom20 and Tom7, displaying different levels of similarity to the annotated Tom proteins and to each other. Interestingly, the Tom proteins of these two species are most similar to Opisthokonta Tom proteins, with the exception of Tom40 being most similar to plant Tom40. Moreover, the identified Tom70 appears to be a homolog of animal/yeast Tom70 subunits.

Results

Purification of the TOM Complex of *Acanthamoeba castellanii* and *Dictyostelium discoideum* by Affinity Chromatography

Isolation of the TOM complexes of *A. castellanii* and *D. discoideum* required various experimental strategies because at the time of the project inception the availability of the genome sequences of the two microorganisms differed. The *D. discoideum* genome sequencing project was completed in 2005 and the obtained data were deposited in the Dictybase database (http://dictybase.org; Eichinger et al. 2005). In contrast, for *A. castellanii* only partially assembled genome sequences were available (http://www.hgsc.bcm.tmc.edu/projects/microbial/microbial-pubPreview.xsp?project_id=163) but during the analysis of the subunit organization of the isolated *A. castellanii* TOM complex, its genome and transcriptome were completely assembled (Buczek et al. unpublished data; Clarke et al. 2013). Consequently, we applied affinity chromatography using antibodies against different subunits of the studied complexes.

As described in Methods, the purified antibody against *A. castellanii* Tom40 (AcTom40 antibody) was crucial for the applied procedure of TOM complex isolation. The proper mitochondrial localization of the protein recognized by the antibody

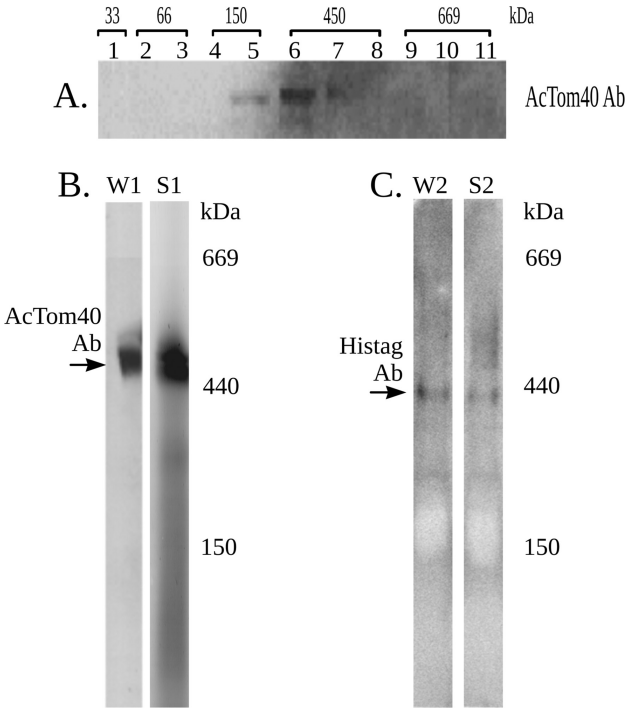

Figure 1. Estimation of the molecular weight of TOM complexes in *Acanthamoeba castellanii* and *Dictyostelium discoideum*. **A**. After solubilization in 1% digitonin the *A. castellanii* mitochondrial outer membrane vesicles (OMVs) were immunoprecipitated on Protein A-Sepharose beads, the eluted fraction was separated on 10-50% linear sucrose gradient, and the consecutive fractions were tested for the presence of Tom40 by an antibody against *A. castellanii* Tom40 (AcTom40 Ab). **B**. Result of BN-PAGE and Western blot of the sucrose gradient fraction containing the highest amount *of A. castellanii* Tom40. **C.** Result of BN-PAGE and Western blot of the *D. discoideum* TOM complex using an antibody directed against 6x His-tag (Histag Ab). S1 and S2, results of BN-PAGE gel staining with Coomasie Brillant Blue; W1 and W2, results of Western blot with proper antisera; M, molecular weight marker. The presented data are typical for 3 independent experiments.

was proven by Western blot in the case of isolated mitochondria and OMVs as well as by immunofluorescence staining performed for intact cells (Supplementary Material Fig. S1). After immunoprecipitation on the Protein A-Sepharose beads, the eluted fraction was separated on a linear sucrose gradient and the obtained consecutive fractions were tested for the presence of Tom40 recognized by the AcTom40 antibody (Fig. 1A). The fraction containing the highest amount of Tom40 (fraction 6) had the molecular weight of about 450 kDa. The molecular weight of the *A. castellanii*

TOM complex was next confirmed by BN-PAGE and Western blot (Fig. 1B), and the result was in agreement with published data (Wojtkowska et al. 2005).

In the case of the *D. discoideum* TOM complex, the tagged version of Tom7 (6x His-Tom7) expressed in the slime mold cells was used to perform the affinity chromatography. Effective expression and proper localization of 6x His-Tom7 was checked by immunofluorescence staining performed for intact cells (Supplementary Material Fig. S1). The isolated OMVs were solubilized with 1% digitonin and the obtained suspension was loaded on Ni-NTA agarose column. Then the eluted fraction was further purified by ion exchange chromatography on a MonoQ column. The molecular weight of the isolated complex of 430 kDa was determined by BN-PAGE and immunodetection, using the antibody against His-tag (Fig. 1C).

Verification of the Identity of the Isolated Complexes by Estimation of Channel Activity

The TOM complex has been shown to form large ion-conducting channels in artificial lipid membranes. At high membrane voltages, the channels of the TOM complex were found to undergo fast transitions from an open state to lower conductance states (e.g. Ahting et al. 1999; Künkele et al. 1998a,b; Muro et al. 2003; Poynor et al. 2008). Therefore, to verify the identity of the isolated complexes, we measured single-channel conductance after reconstitution of the complexes into artificial lipid membranes (BLM system) at membrane potentials of 10 and 70 mV. Typical examples of single-channel insertions of the studied *A. castellanii* and *D. discoideum* complexes (Fig. 2A, B and 2C, D, respectively) indicated the presence of a channel displaying voltage-dependent transition between states of different conductance. The obtained distributions of conductances were then used to build histograms and to calculate values of average conductance of the studied complexes under the given experimental conditions. The presented histograms clearly showed the existence of an open state with conductance of about 2 nS at a membrane potential of 10 mV and in the presence of 1 M KCl. At a membrane potential of 70 mV (in 1 M KCl), transition towards lower conductance states of an average value of about 1 nS was observed for both complexes. Moreover, the fusion protein 1-167 b_2-DHFR, which contains a presequence of mitochondrial cytochrome b_2 (Budzińska et al. 2009), facilitated the transition of channels formed by both complexes from open to lower conductance states when added from the site of positive potentials (Fig. 2 A and C). Thus, the channel activity detected within the complexes isolated from *A. castellanii* and *D. discoideum* OMVs corresponds to the channel activity described for the model TOM complex (e.g. Künkele et al. 1998a,b; Wojtkowska et al. 2005; Poynor et al. 2008). Consequently, the conductance measurements in the BLM system confirmed the identification of the isolated complexes as the TOM complexes of *A. castellanii* and *D. discoideum*. Interestingly, the complexes appeared to display minor differences in the studied properties.

Identification of *A. castellanii* and *D. discoideum* TOM Complex Subunits by Mass Spectrometry and Bioinformatic Tools

To identify subunits of the isolated TOM complexes, an approach encompassing experimental and theoretical methods was applied. Putative subunits of the isolated *A. castellanii* and *D. discoideum* complexes, obtained by SDS-PAGE, were investigated by mass spectrometry (LC-MS/MS). To infer and identify peptide sequences from their fragmentation spectra, we searched the Dictybase (http://dictybase.org) for *D. discoideum*, while for *A. castellanii*, a mitochondrial outer membrane protein database built by us on the basis of available data concerning the *A. castellanii* genome and transcriptome sequences (Buczek et al. unpublished data; Clarke et al. 2013). The lowest score for the analyzed peptides was 20.

As shown in Figure 3, the SDS-PAGE separation patterns of the isolated complexes differed between *A. castellanii* and *D. discoideum*, suggesting different subunit organization. As specificity controls, parallel mock purifications were performed for both *A. castellanii* and *D. discoideum* TOM complexes. Then the bands that differed between control (Mock) and specific samples (TOM) were cut out from the gel and analyzed by LC-MS/MS. The bands corresponding to Tom proteins were marked by arrows with consecutive numbers, whereas the bands identified as non-Tom proteins (which appeared both consistently and inconsistently) were marked with dots (Supplementary Material Table S1).

For the *A. castellanii* TOM complex, the LC-MS/MS data indicated the presence of five Tom proteins. Peptides with the highest score and coverage were obtained for Tom40 and Tom7. The proteins corresponded to the bands of about 40

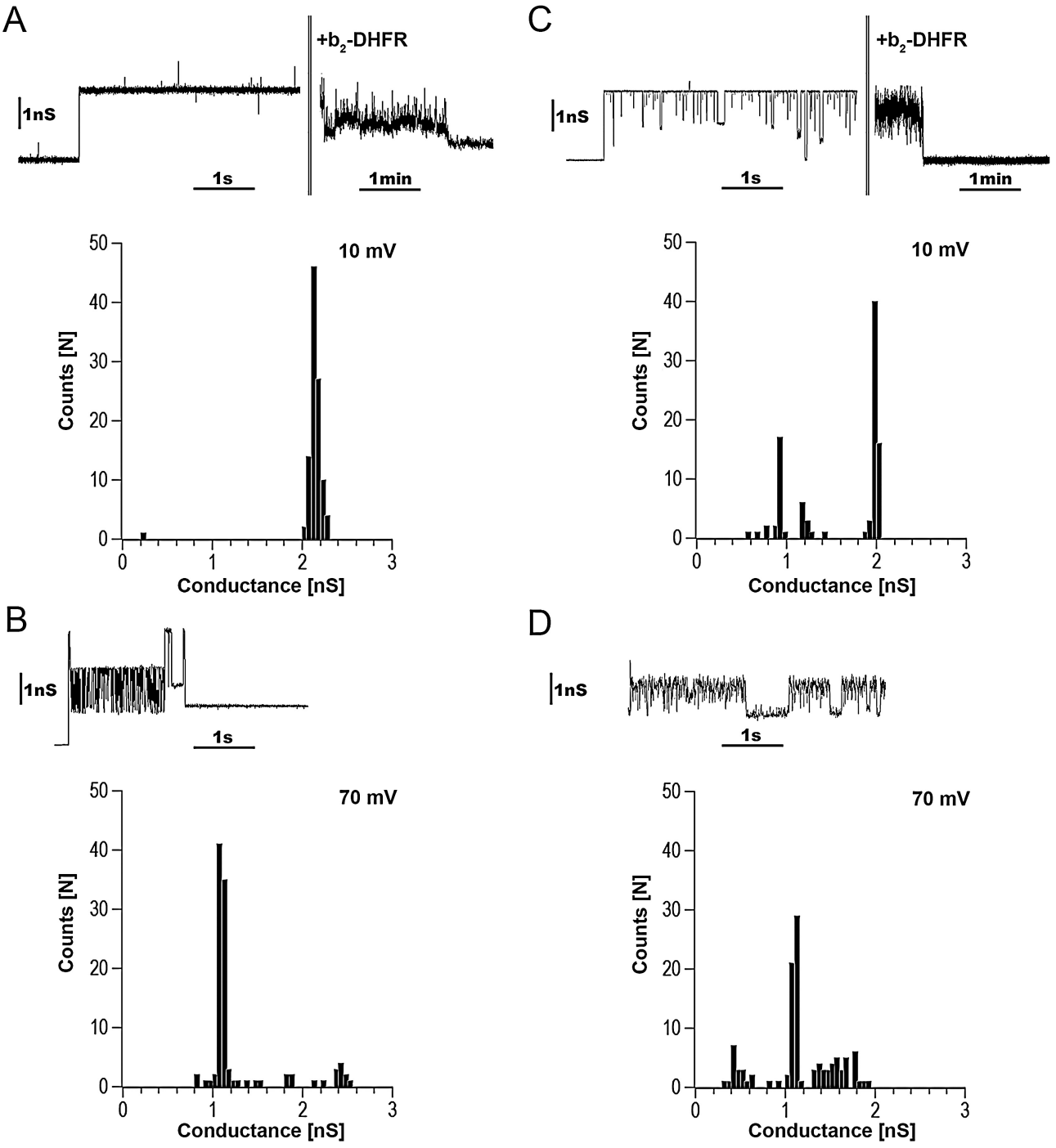

Figure 2. TOM complex channel activity in *Acanthamoeba castellanii* and *Dictyostelium discoideum* after reconstitution into artificial lipid membranes. Examples of single-channel insertions and the created conductance histograms for *A. castellanii* in the presence of 10 and 70 mV (**A** and **B**, respectively) and for *D. discoideum* in the presence of 10 and 70 mV (**C** and **D**, respectively). On average, about 80 insertions for a given complex and applied conditions were analyzed. A and C, present also examples of single-channel activity recorded for the *A. castellanii* and *D. discoideum* complexes, respectively, in the presence of the fusion protein 1-167b_2-DHFR (b_2-DHFR).

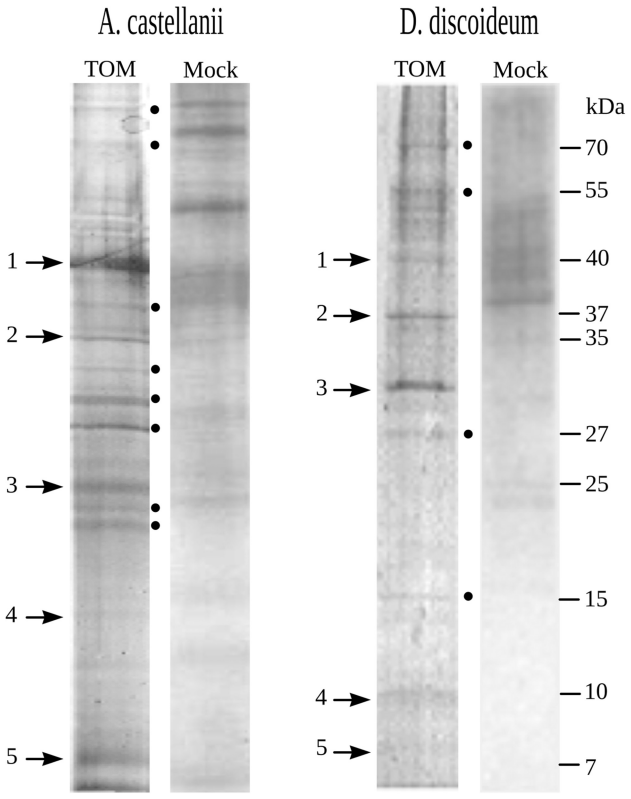

Figure 3. SDS-PAGE separation pattern of the purified TOM complex of *Acanthamoeba castellanii* and *Dictyostelium discoideum*. Arrows and dots indicate Tom and non-Tom proteins, respectively, identified due to LC-MS/MS analysis. The presented data are typical for 5 independent experiments. Mock purification in *A. castellanii* was performed without the antibody against *A. castellanii* Tom40 covalently coupled to A-Sepharose beads, whereas in *D. discoideum* it was performed for a strain with non-His-tagged Tom7.

and 7 kDa, respectively (Fig. 3, bands nos. 1 and 5), and displayed high sequence similarity with cognate proteins of other organisms (Supplementary Material Fig. S2 and Table S2). The complex contained also three other proteins comprising peptides found in bands migrating at about 15, 25 and 35 kDa (Fig. 3, bands nos. 4, 3 and 2, respectively). The *A. castellanii* database search revealed that these peptides were likely part of the proteins deposited under the following accession numbers: XP_004333415.1 (30 kDa), XP_00435349.1 (45 kDa), and XP_004339622.1 (98 kDa). These proteins were further analyzed by BLASTp and we detected a low similarity to known Tom20 and Tom22 as well as much higher similarity to known Tom70, respectively (Supplementary Material Fig. S2 and Table S2). The identified subunits of the *A. castellanii* TOM complex found by the applied approach were listed in Table 1. The obtained results indicated the highest similarity of the identified subunits to the proteins identified for representatives of the Opisthokonta with the exception of the identified Tom40 and Tom 20, which seemed to be most closely related to Tom40 and Tom20 of the Archaeplastida (Plantae). It has been previously shown that the predicted Tom40 sequence of *A. castellanii* is located closely to the plant node of the phylogenetic tree (Wojtkowska et al. 2012). Accordingly, phylogenetic analysis of the identified Tom proteins of *A. castellanii* supported the results of BLASTp analysis and indicated grouping of *A. castellanii* Tom7, Tom22 and Tom70 with Opisthokonta proteins and *A. castellanii* Tom20 with Archaeplastida proteins, whereas *A. castellanii* Tom40 appeared to locate between plant and Opisthokonta proteins (Supplementary Material Fig. S3).

For the *D. discoideum* complex, LC-MS/MS analysis also indicated the presence of five subunits (Fig. 3, marked with arrows). Peptides with the highest score and coverage were obtained for the slime mold Tom7 and Tom40 (Supplementary Material Fig. S4), which corresponded to bands of molecular weight of about 10 and 37 kDa, respectively (Fig. 3, bands nos. 4 and 2). Moreover, three other proteins of unknown function were detected in the complex (Fig. 3, bands nos. 5, 3 and 1, respectively): a protein of about 9 kDa (DDB_G0283573) present in the same band as Tom7, a protein of about 30 kDa (migrating between 27 and 35 kDa; DDB_G0288629), and a protein of about 60 kDa (found in the band at about 40 kDa, DDB_G0275389). The similarity search performed using BLASTp demonstrated a very weak sequence similarity to known Tom20 and Tom22 as well as much higher similarity to known Tom70, respectively (Supplementary Material Fig. S4 and Table S3). The identified subunits of *D. discoideum* TOM are listed in Table 2. Interestingly, as in the case of the *A. castellanii* complex, the identified subunits displayed the highest similarity to the proteins annotated for representatives of the Opisthokonta, with exception of Tom40, which seems to be closest related to the Archaeplastida protein. Accordingly, phylogenetic analysis of the identified Tom proteins of *D. discoideum* supported their grouping with Opisthokonta proteins with the exception of Tom40 (Supplementary Material Fig. S5)

The grouping of A. *castellanii* and *D. discoideum* Tom proteins with cognate proteins of organisms representing other groups than Opisthokonta

Table 1. The most similar sequences for the identified subunits of the *Acanthamoeba castellanii* TOM complex. * Classification based on Cavalier-Smith et al. (2014). **Tom proteins already annotated for *A. castellanii*: AJE29748 (Tom7Ac) and ADZ24223 (Tom40Ac).

Subunit	Length (AA)	The best match	GenBank accession no.	Length (AA)	e-value	Group*
Tom7Ac	64	*Capsaspora owczarzaki* ATCC 30864	EFW44240	69	3.00E-05	Opisthokonta (Choanozoa)
Tom20Ac	273	*Ostreococcus tauri*	CAL57327	206	3.00E-06	Archaeplastida (Plantae)
Tom22Ac	413	*Neosartorya fumigata*	KEY79581	143	1.00E-04	Opisthokonta (Fungi)
Tom40Ac	361	*Tetraselmis* sp. GSL018	JAC84130	333	7.00E-34	Archaeplastida (Plantae)
Tom70Ac	898	*Neurospora crassa*	AAD21979	624	2.00E-15	Opisthokonta (Fungi)

AA, amino acids

(Supplementary Material Figs. S3 and S5 and Tables S2 and S3) is rather surprising, given that the Amoebozoa are regarded to be a sister group of the Opisthokonta, which diverged from the animal/fungal line after its split from plants (e.g. Adl et al. 2005, 2012; Cavalier-Smith et al. 2014; Eichinger et al. 2005; Keeling et al. 2005; Schilde and Schaap 2013). However, this does not exclude that beside animals and fungi, also plants share some genes with the Amoebozoa (Song et al. 2005).

The observed similarity of *A. castellanii* and *D. discoideum* Tom70 to Opisthokonta Tom70 contradicts the previously published conclusion that no protein similar to Tom70 in domain architecture, beyond the simple presence of tetratricopeptide repeat (TPR) motifs, exists outside the animal/fungal lineage (Chan et al. 2006). Consequently, we decided to compare the domain distribution within the identified Tom70 proteins with model Tom70 from *Homo sapiens*, *Rattus norvegicus*, *Neurospora crassa*, and *Saccharomyces cerevisiae* (Supplementary Material Fig. S6). Importantly, both *A. castellanii* and *D. discoideum* Tom70 displayed the location of the transmembrane domain (TM) and distribution of eleven TPR motifs analogous to those obtained for model Tom70. Thus the characteristic domain organization of Tom70 appears to be not confined to animals and fungi. Hence, the obtained results indicated the presence of five subunits in the *A. castellanii* and *D. discoideum* complexes, namely Tom70, Tom40, Tom22, Tom20, and Tom7, although the presence of Tom22 and Tom20 was

Table 2. The most similar sequences for the identified subunits of the *Dictyostelium discoideum* TOM complex. * Classification based on Cavalier-Smith et al. (2014). **Tom proteins already annotated for *D. discoideum*: XP_639342 (Tom7Dd) and XP_642798 (Tom40Dd).

Subunit	Length (AA)	The best match	GenBank accession no.	Length (AA)	e-value	Group*
Tom7Dd	55	*Polysphondylium pallidum*	EFA78398*	54	7.00E-08	Amoebozoa
Tom20Dd	76	*Rhizoctonia solani*	CCO31903	388	6.00E-03	Opisthokonta (Fungi)
Tom22Dd	263	*Arthroderma gypseum*	E4UYQ6-1	159	2.00E-02	Opisthokonta (Fungi)
Tom40Dd	314	*Chlorella variabilis*	EFN52297	313	1.00E-18	Archaeplastida (Plantae)
Tom70Dd	535	*Schizosaccharomyces pombe*	O14217	625	8.00E-13	Opisthokonta (Fungi)

AA, amino acids, * Makiuchi et al. 2013

not strongly supported. Accordingly, sequence multiple alignments (Supplementary Material Fig. S7) indicated a high similarity of *A. castellanii* Tom70, Tom40 and Tom7, whereas the similarity for Tom20 and Tom22 was much less pronounced.

We also analyzed the non-Tom proteins detected for both isolated *A. castellanii* and *D. discoideum* TOM complexes (Supplementary Material Table S1). The non-Tom proteins that occurred consistently included some proteins of the inner membrane (metabolite carriers, subunits of ATP synthase, subunits of the respiratory chain), some chaperones and proteins of the outer membrane, including VDAC, and subunits of the TOB/SAM complex. Interestingly, for *A. castellanii*, but not for *D. discoideum*, subunits of the ERMES complex were detected (e.g. Flinner et al. 2013). On the one hand, the presence of these proteins in the isolated TOM complexes may result from OMV contamination with the inner membrane proteins but also from interaction of the TOM complex with other mitochondrial proteins. For example, the interaction of the TOM complex with the TOB/SAM complex (Qiu et al. 2013) appeared to be supported by results of this study. On the other hand, the difference concerning the presence of the ERMES complex subunit constitutes an interesting observation, as it is known that the complex interacts with the TOB/SAM complex.

Discussion

The TOM complex consists of subunits formed by proteins shared by all eukaryotes and additional subunits that have been added over time and are specific to a particular eukaryotic lineage (e.g. Dolezal et al. 2010). Available data indicate that subunit organization of the TOM complex of animals and plants is more or less similar to that described for *Saccharomyces cerevisiae* and *Neurospora crassa* and is considered as the canonical one (Murcha et al. 2014; Sokol et al. 2014), while in the case of other eukaryotes the differences are more pronounced.

The Amoebozoa, one of the presently proposed systematic groups, is poorly sampled for mitochondrial protein import complexes, although the applicable data indicate that the TOM complex subunit organization of the organisms may differ distinctly from the canonical one. For example, the TOM complex of *Entamoeba* spp. contains only Tom40 and probably Tom60, defined as a receptor protein (Dolezal et al. 2010; Likic et al. 2010; Lithgow and Schneider 2010; Makiuchi et al. 2013). It should, however, be remembered that entamoebae are mainly pathogenic species with mitochondria-like organelles (mitosomes) that display a dramatic reduction of subunit contents of the protein import complex (e.g. Heinz and Lithgow 2013). On the other hand, it has been suggested that the TOM complex of *Dictyostelium discoideum* consists of Tom40, Tom7, and Tom22 (Barth et al. 2007; Dolezal et al. 2010; Maćasev et al. 2004), whereas the presence of Tom40, Tom7, and Tom70 has been proposed for the complex of *Acanthamoeba castellanii* (Makiuchi et al. 2013; Wojtkowska et al. 2005, 2012). At present, the availability of the *A. castellanii* and *D. discoideum* genomes and transcriptomes (Clarke et al. 2013; www.dictybase.com; Buczek et al. unpublished data) makes it possible to combine sequence analyses in silico with mass spectrometry data to verify subunit organization of the organism TOM complexes.

Interestingly, both TOM complexes appear to have a similar molecular weight. As estimated by BN-PAGE (Fig. 1), the molecular weight is about 450 kDa for the *A. castellanii* complex and about 430 kDa for the *D. discoideum* complex. This is in agreement with published data concerning the TOM complex of *A. castellanii* (Wojtkowska et al. 2005) as well as the TOM complexes of model organisms, such as *N. crassa* and *S. cerevisiae* (e.g. Ahting et al. 2001; Meisinger et al. 2001). Moreover, both complexes reconstituted in artificial lipid membranes, display channel properties characteristic for the TOM complex. It indicates that we observed the existence of an open state with conductance of about 2 nS at a membrane potential of 10 mV (in the presence of 1 M KCl) and transition towards a lower conductance state of an average value of about 1 nS at a membrane potential of 70 mV (Ahting et al. 2001; Künkele et al. 1998b; Meisinger et al. 2001), although the studied complexes differ slightly in the analyzed properties (Fig. 2). The differences might be caused by the different methods of their isolation or may result from differences in subunit organization of the complexes.

The subunit organization of both complexes appears to be similar, as they both contain five subunits confirmed by mass spectrometry data (Tables 1 and 2), although the SDS-PAGE separation patterns of the isolated complexes show differences between the proteins involved (Fig. 3). In both cases the peptides with the highest score and coverage were obtained for Tom40 and Tom7, but the identified proteins differ in molecular weight (Tables 1 and 2) and amino acid sequences (Supplementary Material Figs S2 and S4). However,

such differences concern also other evolutionarily related organisms, such as yeasts and humans (e.g. Chan et al. 2006, Supplementary Material Fig. S4). Differences in molecular weight and amino acid sequences were also observed in the case of putative Tom20, Tom22 and Tom70. Importantly, the e-values obtained for the putative Tom20 and Tom22 similarity search (Tables 1 and 2; Supplementary Material Tables S2 and S3) do not strongly support the presence of both proteins in the TOM complexes of *A. castellanii* and *D. discoideum*. Moreover, neither Tom5 nor Tom6 have been detected for the TOM complexes. Since the complexes undoubtedly contain Tom7, the absence of Tom5 and Tom6 is not caused probably by the resolution of SDS-PAGE, although Tom5, Tom6 and Tom7 do not separate well on SDS-PAGE gels (e.g. Mager et al. 2010). However, it is known that the small size of Tom5 and Tom6 makes their identification challenging and they are present in many but not all lineages (Hewitt et al. 2011). Thus, taking into account that Tom5 and Tom6 are very short proteins and that their sequences are poorly conserved (Smith et al. 2007), we cannot exclude. The TOM complex organization does not correspond to differences in *A. castellanii* and *D. discoideum* life style. The analyzed Neff strain of *A. castellanii* represents a non-pathogenic, free-living and unicellular protozoan, whereas *D. discoideum* displays a variety of phenotypes, including motile unicellular and multicellular stages within the life cycle (e.g. Wojtkowska et al. 2012).

Interestingly, nearly all the proteins identified for the studied TOM complexes display significant similarity to the cognate Opisthokonta proteins (Table 1 and 2; Supplementary Material Tables S2 and S3) and group with the proteins within phylogenetic trees (Supplementary Material Figs S3 and S5). However, the predicted *A. castellanii* Tom20 and Tom40 of both *A. castellanii* and *D. discoideum* seem to be the most similar to the proteins of the Archaeplastida (Plantae). Accordingly, it has been already shown that predicted sequences of *A. castellanii* and *D. discoideum* Tom40 locate basally to the Archaeplastida branches in the phylogenetic tree (Wojtkowska et al. 2012). The latter observation is interesting from the evolutionary point of view, as it suggests a selective pressure on preservation of plant-type Tom40 among animal/fungi-type Tom proteins. This suggestion does not contradict the hypothesis that the divergence of the Amoebozoa from the Opisthokonta is a later event than the divergence of plants from that lineage (Song et al. 2005). Nevertheless, since bootstrap values were rather low (Supplementary Material Figs S3 and S5) these groupings are not robustly supported, hence not very reliable. The same applies to other Tom proteins of *A. castellanii* and *D. discoideum*. This is most likely the result of a low sequence similarity between the analyzed proteins and consequently the very weak phylogenetic signal. The dissimilarity is particularly observed for Tom7 and Tom22 of *D. discoideum*, Tom20 of *A. castellanii* and Tom70 of both *D. discoideum* and *A. castellanii*.

The identification of *A. castellanii* and *D. discoideum* Tom70, displaying significant similarity to the Opisthokonta protein (Supplementary Material Fig. S6) confirms our previously published data concerning the presence of a homolog of animal/yeast Tom70 in the TOM complex of *A. castellanii* (Wojtkowska et al. 2005). However, the observation is inconsistent with the proposed absence of animal/fungal Tom70 in plants and protists (Chan et al. 2006) although the presence of the protein has been proposed for unicellular opisthokonts, stramenopiles and haptophytes (Tsaousis et al. 2011). Our new data clearly indicate the presence of a transmembrane domain at the N terminus of the analyzed proteins and eleven TPR motifs distributed along the amino acid sequence in a way characteristic for the animal/fungal Tom70. Yet, the identified Tom70 of *A. castellanii* (898 amino acids) and *D. discoideum* (535 amino acids) differ distinctly in size from the canonical Tom 70 of animals and fungi (usually 590-625 amino acids). However, it has been shown that even in the case of fungi and animals, proteins identified as Tom70 may contain a number of amino acids different from the usual one. For example, Tom70 of *Encephalitozoon cuniculi* (a fungus) and of *Camelus ferus* (an animal) have been reported to contain 477 and 504 amino acids, respectively (Waller et al. 2009; Jirimutu et al. 2012, respectively). Moreover, the presence of Tom70 of molecular weight of 93.2 kD (833 amino acids) has been shown for *Blastocystis sp.* (Chromalveolata). Nevertheless, we cannot exclude inaccuracies in *A. castellanii* and *D. discoideum* genome assembly. Interestingly, peptides corresponding to Tom70 of *A. castellanii* and *D. discoideum* were detected for proteins of distinctly lower molecular weights (Fig. 3). However, Tom70, as the peripheral component of the TOM complex, largely dissociates from the complex upon solubilization with digitonin (e.g. Meisinger et al. 2001; Reinhold et al. 2012) and may undergo degradation. Accordingly, when we performed an analysis of *A. castellanii* and *D. discoideum* Tom70 using untreated mitochondria, the bands corresponding to the Tom proteins were found to migrate at about

89 and 60 kD, respectively (Supplementary Material Figs S2 and S4).

As mentioned above, our data undoubtedly confirm the presence of Tom7 in the TOM complex of *A. castellanii* and *D. discoideum* (Makiuchi et al. 2013; Maćasev et al. 2004, respectively). Tom20 and Tom22 candidates are supported by the database search. The alignments of these proteins within the top sequences have similar e-values as in the case of Tom7 database search, particularly in the case of *A. castellanii* proteins (Tables 1 and 2, Supplementary Material Tables S2 and S3). It should be also remembered that the presence of Tom22 was postulated for the *D. discoideum* TOM complex (e.g. Dolezal et al. 2010). Furthermore, alignments of sequences with a recent shared ancestry that display a high degree of similarity have very low e-values, whereas sequences with an ancient common ancestry are deeply divergent, with larger e-values in the database search (Kerfeld and Scott 2011). Thus our results appear to support the presence of Tom20 and Tom22 in the TOM complexes of *A. castellanii* and *D. discoideum*. The low similarity between Tom20 and Tom22 of *A. castellanii* and *D. discoideum* (Supplementary Material Fig. S7) as well as the higher similarity of the other identified Tom proteins may suggest different rates of Tom protein evolution within the Amoebozoa (e.g. Wall et al. 2005) although Song et al. (2005) suggested that the rates of protein evolution in the Amoebozoa are comparable to those of the plants and animals.

Conclusion

Our results indicate the presence of five subunits in the TOM complex of *A. castellanii* and *D. discoideum*, namely Tom70, Tom40, Tom22, Tom20 and Tom7. Nearly all the proteins display remarkable similarity to the cognate Opisthokonta proteins, with the exception of the identified Tom40 being the most similar to the plant Tom40. Interestingly, the identified Tom70 appears to be a homolog of animal/yeast Tom70. However, the presence of Tom20 and Tom22, particularly in the case of *D. discoideum*, is less supported by the presented data. Further experiments may be necessary to understand fully the structure of the TOM complex in the Amoebozoa.

Methods

Growth conditions, isolation of mitochondria and the outer membrane vesicles of *Acanthamoeba castellanii* and *Dictyostelium discoideum*: The amoeba *A. castellanii* (strain Neff) was cultured as described by Jarmuszkiewicz et al. (1997). Trophozoites of the amoeba were collected at the exponential growth phase (46-48 h after inoculation), at the density of approximately 4-5 $\times$ 10^6 cells/ml. *A. castellanii* mitochondria were isolated according to the procedure described by Jarmuszkiewicz et al. (1997). The slime mold *D. discoideum* (strain AX2, kindly provided by Prof. Michael Schleicher, LMU Muenchen) was cultured at 19 °C in liquid or solid HL5 medium. The cells were collected at the exponential growth phase (14-16 h after inoculation), at the density of approximately 0.5-1.0 $\times$ 10^6 cells/ml. *D. discoideum* mitochondria were isolated according to the procedure described by Czarna et al. (2010). The mitochondrial outer membrane vesicles (OMVs) were isolated and purified as described by Wojtkowska et al. (2005).

Antibody production and immunofluorescent staining: The antibody against *A. castellanii* Tom40 was supplied by Pineda Company (Berlin, Germany) using the synthetic peptide: NH_2-CFNMELDMKGSDYQAQFKWH-$CONH_2$. The provided serum was purified on modified sulfhydryl agarose (Pierce) according to the manufacturer's instructions. For *D. discoideum*, the commercially available antibody against 6x His tag (Qiagen) was applied. The specificity of both antibodies was tested by immunodecoration of Western blots and immunofluorescence staining. In the case of the latter method, to visualize mitochondria, cell suspensions were incubated with 200 nM MitoTracker Red (Invitrogen) for 1 h at 37 °C. Then, the cells were washed in phosphate buffered saline (PBS), fixed, and incubated for 1 h at room temperature with goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC) (Santa Cruz Biotechnology) at a dilution of 1:100, as described by Slocinska et al. (2011). Microscopic images were recorded using a Nikon inverted fluorescence microscope.

Expression of 6x His-Tom7 in *Dictyostelium discoideum* cells: The *D. discoideum* Tom7 cDNA (Morio et al. 1998; Urushihara et al. 2004, 2006) was amplified by polymerase chain reaction (PCR) using the sense primer, 5′-TAGGGATCCATGGAAATGGAAAAATATTACGAATA-3′, with the BamHI restriction site (underlined), and the antisense primer, 5′-CTAGTCTAGATTATTGTTCCATAATTCCTAATGGACC-3′, with the XbaI restriction site (underlined). The PCR product was cloned into the expression vector pDXA-3H (Manstein et al. 1995) and used for *D. discoideum* cell transformation. Namely, the cells (about 5 $\times$ 10^6/ml) were incubated for 15 min on ice, pelleted by centrifugation at 500 *g* for 5 min at 4 °C, and washed twice with ice-cold Sorensen's phosphate buffer. Then, the cell suspension (about 10 $\times$ 10^6 cell/ml) in electroporation buffer (10 mM potassium phosphate, pH = 6.1, 50 mM glucose) was transferred to cold 0.4 cm electroporation cuvette and electroporated (Electroporator XCell, Biorad) at 1 kV per 10 μF and 1 ms, twice with about 5 s between pulses. After cooling the cuvette on ice for 5 min, 100 mM $CaCl_2$ and 100 mM $MgCl_2$ were added to a final concentration of 1 mM each and the cells were incubated for 15 min at 20 °C to heal. The cells were transferred to a Petri dish containing 12 ml of HL5 and cultured overnight at 20 °C. The transformants were selected by spreader dilutions on lawns of non-pathogenic Klebsiella aerogenes and then by addition of 10 μg/ml of G418 (Sigma-Aldrich). The resistant colonies were isolated as described by Fey et al. (2007).

Purification of the *Acanthamoeba castellanii* TOM complex: We solubilized 1 mg of the *A. castellanii* mitochondrial OMVs in 1 ml of ice-cold solubilization buffer (20 mM Tris/Cl, pH 7.4, 0.1 M EDTA, 50 mM NaCl, 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride, PMSF) containing 1% (w/v) digitonin for 20 min at 4 °C. After a clarifying spin for 20 min at 20, 000 *g*, the

supernatant was incubated overnight with protein A-Sepharose beads, with the antibody against *A. castellanii* Tom40 (AcTom40 antibody) covalently coupled by dimethyl pimelimidate (DMP) and without the antibody (mock purification). The resin was washed with 10 ml of the solubilization buffer containing 0.2% (w/v) digitonin and 1 mM PMSF, and the bound proteins were eluted with 2 × 1 ml of 0.1 M glycine, pH 3.0 and neutralized immediately by adding 1M Tris/Cl pH 8.8 to a final concentration of 100 mM. Next, 1 ml of the eluted fraction was dialyzed against 5 mM 3-(N-morpholino)propanesulfonic acid (MOPS), pH 7.4 and separated on 10–50% linear sucrose gradient using centrifugation at 220, 000 *g* for 16 h. Each fraction was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, see below) and subsequently immunodecorated by AcTom40 antibody.

Purification of the *Dictyostelium discoideum* TOM complex: Similarly, 1 mg of OMVs of *D. discoideum* strain expressing 6x His-Tom7 (and strain with non-tagged Tom7 for mock purification) were solubilized in 1 ml of ice-cold buffer A (50 mM NaH_2PO_4, 300 Mm NaCl, 10 mM imidazole pH 8.0, 10% glycerol, and 1 mM PMSF) containing 1% (w/v) digitonin for 20 min at 4 °C. After a clarifying spin (20 min, 15, 000 *g*), the supernatant was loaded on Ni-NTA–agarose (HiTrap Affinity column GE Healthcare). The resin was then washed with 10 volumes of buffer A containing 0.05% digitonin and subsequently with five volumes of lineal imidazole gradient, using the same buffer but with 300 mM imidazole. The bound proteins were eluted with 150 mM imidazole and the eluted fraction was dialyzed against buffer B (50 mM potassium acetate, 10 mM MOPS, pH 7.0, 20% glycerol, 1 mM PMSF, and 0.1% digitonin) and afterwards loaded on anion-exchange MonoQ column (Pharmacia Biotech). The loaded material was then eluted by a linear 0–500 mM KCl gradient in the same buffer.

Conductance measurements of single channels in black lipid membranes (BLM system): Artificial phospholipid membranes were formed by the painting technique from soybean asolectin (Avanti Polar Lipids, Alabaster, AL) suspended at a concentration of 25 mg/ml in n-decane, across a circular hole (aperture diameter about 250 mm) in the thin wall of Delrin chamber (Warner Instruments) separating two compartments (*cis*–*trans*) filled with unbuffered 1 M KCl, pH 7.0 (Karachitos et al. 2009). The chamber was connected to the recording equipment through a matched pair of Ag–AgCl electrodes. About 1–2 μg of TOM complex preparations were added to the *cis* compartment after membrane formation. *Cis* also refers to the compartment where the voltage was held. Signals were amplified by BLM-120 bilayer amplifier (Bio-LOGIC Science Instruments) and computer software was used for data collection. On average, 5 insertion events for a given condition and TOM complex were analyzed. When indicated, the measurements were performed in the presence of the fusion protein 1-167b_2-DHFR (a kind gift of Małgorzata Budzińska) applied at a concentration of 5 μg/ml.

Blue Native/ Polyacrylamide Gel Electrophoresis (BN-PAGE):BN-PAGE was performed in 4% stacking gels followed by 6.0–16.5% native gradient separating gels prepared in 1.5 M e-amino-n-caproic acid and 0.15 M Bis-Tris, pH 7. The protein samples (100 μg) were mixed with SERVA Sample Buffer for Blue Native (2x). SERVA Native Marker, Liquid Mix for BN/CN was applied as a molecular weight marker. The running buffers were the anode buffer (50 mM Bis–Tris, pH 7.0) and the blue cathode buffer (15 mM Bis–Tris, 50 mM Tricine and 0.02% Serva Blue). Electrophoresis was performed at 15 mA for approximately 2.5-3.0 h at 4 °C, and stopped when the tracking line of Serva Blue dye had reached 3/4 of the separating gel.

Bioinformatic tools: Sequences of all known Tom proteins were downloaded from the NCBI (http://www.ncbi.nlm.nih.gov/) and Pfam (http://pfam.sanger.ac.uk/) databases and used as queries in BLASTp (Altschul et al. 1990) searches against the *A. castellanii* genome (Clarke et al. 2013) and transcriptome (Buczek et al. unpublished data) as well as against the *D. discoideum* genome (www.dictybase.com). The transmembrane domain prediction was performed by TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) and TMHMM (http://www.cbs.dtu.dk/services/TMHMM/), whereas TPR motives were predicted with TPRpred (http://toolkit.tuebingen.mpg.de/tprpred) (Karpenahalli et al. 2007). Multiple sequence alignments were calculated with Muscle (3.8) (Edgar 2004) (http://www.ebi.ac.uk/Tools/msa/muscle/). Unrooted phylogenetic trees were calculated using RAxML 7.0.4 with default parameters and 1000 bootstraps (Stamatakis et al. 2008). To visualise and edit the obtained phylogenetic trees, FigTree version 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/) and Inkscape (www.inkscape.org) was used.

Other methods: Protein concentrations were measured by the method of Bradford, and albumin bovine serum (essentially fatty acid free) was used as a standard. After SDS-PAGE, the gels were stained with silver and Coomassie Brilliant Blue G-250, and blotted by the semi-dry transfer method. The studied proteins were visualized by the ECL Western Blotting System (GE Healthcare) after immunodecoration. Analysis by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was performed in the Laboratory of Mass Spectrometry, Institute of Biochemistry and Biophysics, Polish Academy of Sciences (Warsaw, Poland). The obtained data were analyzed automatically by matching against the prepared *A. castellanii* database (composed of protein sequences found by BLASTp) and the NCBI protein database using Mascot Search (http://www.matrixscience.com).

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Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.protis.2015.05.005.

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